



Picogram per liter quantification of pyrethroid and organophosphate insecticides in surface waters: a result of large enrichment with liquid–liquid extraction and gas chromatography coupled to mass spectrometry using atmospheric pressure chemical ionization

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Abstract

Insecticides such as pyrethroids and organophosphates are extensively used globally. Once released into surface water bodies, they can pose a major threat to aquatic ecosystems already at trace concentrations. Therefore, selected pyrethroids and organophosphates are listed as priority substances within the European Water Framework Directive with chronic quality criteria in the picogram per liter range. Previously applied analytical methods were unable to detect pyrethroids and organophosphates at ecotoxicological relevant concentrations, thereby hindering the assessment of surface water quality. In this work, we developed an ultra-sensitive method for the analysis of 12 pyrethroid and two organophosphate insecticides in surface waters. This method is based on the liquid–liquid extraction of surface water samples with *n*-hexane to achieve large enrichment factors (4000×) and subsequent chemical analysis by gas chromatography coupled to tandem mass spectrometry using atmospheric pressure chemical ionization, a soft ionization technique. Quality control parameters including the method limits of quantification (12.5–125 pg L⁻¹), intra-day precision (1–22%), intra-day accuracy (84–133%), and absolute recoveries covering liquid–liquid extraction (67–114%) showed that the method is sensitive and robust and therefore suitable for the analysis of pyrethroids and organophosphates in surface waters. The developed method was applied to Swiss surface water samples and detected pyrethroids and organophosphates below the ecotoxicological relevant concentrations, exemplifying the suitability of the proposed method for aquatic monitoring.

Keywords Insecticides · Atmospheric pressure chemical ionization · Gas chromatography coupled to mass spectrometry · Surface water · Picogram per liter quantification · Liquid–liquid extraction

Introduction

In areas with mainly agricultural land use, pesticides can enter surface waters, thereby adversely affecting aquatic

ecosystems. Pesticides, notably insecticides, pose a high ecotoxicological risk to aquatic organisms [1, 2]. One of the most toxic insecticide classes towards aquatic organisms are pyrethroids and non-polar organophosphates. Even in the low nanogram per liter range, these compounds can negatively affect crustaceans with amphipods being one of the most sensitive taxa [3–5]. Pyrethroids can exhibit one, two, or three chiral centers leading to two, four, or eight stereoisomers, respectively [6]. The toxicity and the environmental behavior such as degradation of stereoisomers can vary, and the toxic potential of pyrethroids often only refers to single isomers [7]. As an example, the toxicity of the pyrethroid cypermethrin (see Fig. 1a) only stems from two (1R-cis-αS and 1R-trans-αS) out of eight stereoisomers, whereas the remaining stereoisomers are much less active [8, 9]. Since these differing

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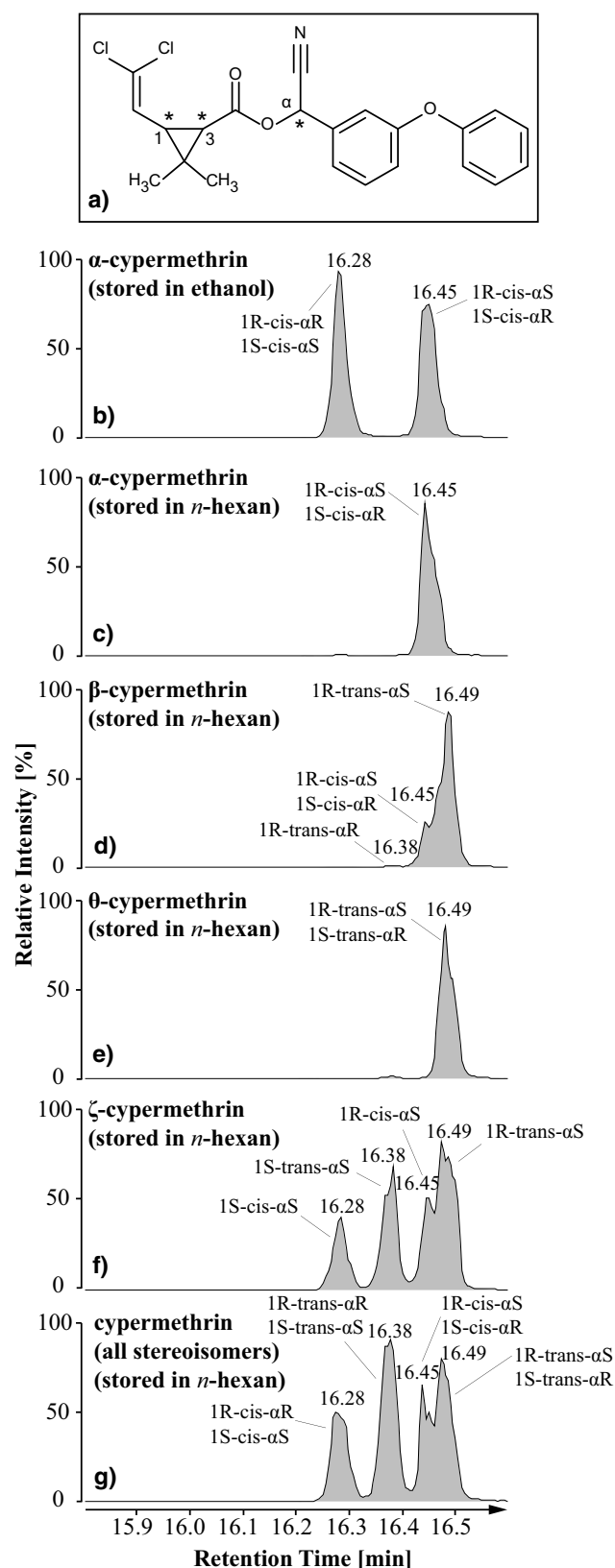


Fig. 1 MRM chromatograms of α-cypermethrin (**b**, **c**), β-cypermethrin (**d**), θ-cypermethrin (**e**), ζ-cypermethrin (**f**), and of cypermethrin including all stereoisomers (**g**) using an apolar fused silica Rtx-5MS GC column. All separated peaks are labeled with the underlying stereoisomers. Note that a different GC column of the same type was used for the measurement of the environmental samples and calibration standards displayed in Fig. 2, explaining why the retention times of the cypermethrin peaks are slightly shifted

isomers [10]. However, toxicity testing of pyrethroids is predominantly based on isomer mixtures that is why available environmental quality standards (EQSs) mostly refer to the mixture of all stereoisomers. Therefore, within the European Water Framework Directive (WFD), the pyrethroid cypermethrin (as a mixture of all stereoisomers) is listed as a priority substance with a chronic quality criterion (AA-EQS: annual average environmental quality standard) in surface waters of 80 pg L^{-1} based on the total water concentration (see Table 2) [11]. In Switzerland, the Swiss Center for Applied Ecotoxicology proposed EQSs for selected pyrethroids and organophosphates in the picogram per liter range (see Table 2) that are to be included into the water protection ordinance in the future [12]. Thus, to assess the water quality with respect to long-term ecotoxicological effects, a highly sensitive and selective analytical method is required to detect pyrethroid and organophosphate insecticides below their respective AA-EQSs (in the picogram per liter range).

EQSs for the selected insecticides are based on the total water concentration that comprises the dissolved fraction and the particle/dissolved organic matter (DOM) bound fraction. Since pyrethroids are highly hydrophobic ($\log K_{ow} \sim 3\text{--}7$, see Table 2), they tend to sorb strongly to particles or to DOM and thus exhibit low water solubilities (few micrograms per liter) [13, 14]. Liu et al. [15] have shown that only 0.4 to 1% of selected pyrethroids are present in the freely dissolved phase in streams. Therefore, many analytical methods focus on the detection of pyrethroids in sediments [16]. However, pyrethroid toxicity towards epibenthic or pelagic aquatic organisms such as filter feeders is not only dependent on the bioavailable dissolved fraction since particle/DOM associated pyrethroids can be taken up, thereby contributing to the overall toxicity. Accordingly, to determine the total fraction of dissolved and particle/DOM bound pyrethroids, the analysis of unfiltered water samples is required. Most common extraction and enrichment methods of pyrethroids from unfiltered water samples include liquid–liquid extraction (LLE) and solid-phase extraction (see reviews by Albaseer et al. [17] and Feo et al. [16]).

Although some methods using liquid chromatography (LC) [18, 19] were developed for the analysis of pyrethroids in water samples, gas chromatography (GC) coupled to electron capture detection, to single mass spectrometry (MS) or to tandem MS has been predominantly applied for the detection of pyrethroids in water samples [16]. Using conventional GC

insecticidal activities are known for some pyrethroid isomers, isomer-enriched products have been manufactured (e.g., α- and ζ-cypermethrin) which often only include the most potent

columns with materials such as fused silica, diastereomeric selectivity of pyrethroids can be achieved, whereas enantiomeric pairs are usually not separated [7, 20]. Enantiomeric separation can be obtained with chiral GC columns but because of the lack of single enantiomer reference standards, a distinct assignment of all stereoisomers is often not possible. However, in the context of EQSs that predominately refer to isomer mixtures, a distinction of single stereoisomers is not mandatory. GC/MS using the hard ionization technique of electron ionization (EI) leads to a strong fragmentation of pyrethroids. The obtained fragments are often unspecific and of low intensity, thereby compromising sensitivity and selectivity [16]. When using GC tandem MS (triple quadrupole) in combination with soft ionization techniques such as chemical ionization (CI) [21, 22] or atmospheric pressure chemical ionization (APCI) [23, 24], the sensitivity and selectivity can be highly improved. Soft ionization techniques reduce fragmentation and often produce molecular ions in high abundance.

Method limits of quantification (MLOQs) of published studies analyzing total concentrations of pyrethroids in water samples are in the nanogram per liter range for selected analytes (from several up to several hundred nanograms per liter), thereby not reaching the required AA-EQSs (see Table 2) for ecotoxicological risk assessment of pyrethroids [18, 19, 25–28]. These methods are lacking adequate sensitivities either because of hard ionization techniques such as EI [28], the choice of the detector (electron capture detector or high resolution MS versus triple quadrupole MS) [25–27], or the choice of the chromatographic system (LC versus GC) [18, 19]. Only one study reports MLOQs for a limited number of pyrethroids in unfiltered surface water samples in the sub-nanogram per liter range ($\sim 0.2 \text{ ng L}^{-1}$) using ultrasound-assisted emulsification–extraction and detection by GC-negative CI-MS/MS [22].

Moschet et al. [29] developed a GC-EI-MS/MS method for the analysis of pyrethroids and organophosphates in surface water that reaches MLOQs in the picogram per liter range ($20\text{--}400 \text{ pg L}^{-1}$). However, this method uses passive sampling and thereby only considers the dissolved fraction. Consequently, the developed method does not fulfill the requirements of EQSs given within the WFD based on total water concentrations. In addition, the lack of substance-specific sampling rates hinders a reliable quantification leading to highly uncertain concentration estimates. Hence, an analytical method for detecting the total fraction of pyrethroids and organophosphates in water samples at ecotoxicological relevant concentrations is missing, thereby enabling the assessment of existing AA-EQSs.

Therefore, the goal of this study was to develop an ultra-sensitive and time- and cost-efficient method for the direct extraction of pyrethroids and organophosphates from unfiltered surface water samples with MLOQs below their

corresponding AA-EQSs. The analytical strategy was to apply LLE with large enrichment factors and sensitive analysis by GC-APCI-MS/MS following the APCI optimization by Portoles et al. [23]. The method should allow for the detection and quantification of 12 relevant and frequently used apolar pyrethroids (see $\log K_{ow}$ in Table 2) (acrinathrin, bifenthrin, α -cypermethrin, deltamethrin, empenethrin, esfenvalerate, etofenprox, τ -fluvalinate, λ -cyhalothrin, permethrin, phenothrin, tefluthrin) and two organophosphates (chlorpyrifos, chlorpyrifos-methyl) in the low picogram per liter range in surface water samples. Three more polar pyrethroids (see $\log K_{ow}$ in Table 2) (allethrin, imiprothrin, tetramethrin) were included into the analysis to evaluate the limitations of the method regarding more polar analytes. Additionally, a focus was given to the fact that many pyrethroids exist and are applied as a mixture of stereoisomers. Finally, the developed method was validated and applied to Swiss surface water samples.

Materials and methods

Chemicals and solutions

Single analyte standard solutions and isotopically labeled internal standard (ISTD) solutions were prepared in ethanol. Analyte standard mix and ISTD mix solutions were prepared in acetonitrile to ensure a proper mixing of the water sample, spiked analytes, and ISTDs. Detailed information about all chemicals and solutions used during sample preparation and instrumental analysis are provided in the [Electronic Supplementary Material \(ESM\) A](#).

Sampling site and sampling of surface water

Time-proportional 3.5-day composite surface water samples were taken from a small stream (stream order of 1 according to Strahler [30]) located in the Swiss Plateau. The catchment size was 2.8 km^2 , and the land use in the catchment was divided into arable land (54%), forest (33%), urban area (6%), grass land (5%), orchards (0.3%), and vegetables (0.2%). Samples were collected between March and October 2017. The samples were accumulated by a permanently installed automatic sampling device (Maxx portable sampler TP5 C, cooled on-site at 4°C). The composite samples consisted of 112 subsamples of 35 mL each that were automatically taken every 45 min and stored in 4-L glass bottles.

Sample preparation of surface water by liquid–liquid extraction

Surface water samples were stored in glass bottles at -20°C . After unfreezing, bottles were thoroughly shaken for 2 min. In

total, 200 mL sample aliquots of unfiltered surface water were transferred into 200 mL glass volumetric flasks and 50 μL ISTD mix solution composed of bifenthrin-d5, chlorpyrifos-methyl-d6, trans-cypermethrin-d6, etofenprox-d5, fenvalerate-d7 (each 0.005 mg L^{-1}), deltamethrin-d5, and atrazine-d5 (each 0.05 mg L^{-1}) was added. Water samples were stirred for 15 min with 5 mL of *n*-hexane on a magnetic stirrer plate (990 rpm) using glass-coated magnetic stirrers. The organic phases including the emulsion between the two phases were transferred into 10 mL glass centrifuge vials. Water samples were stirred again with 5 mL of *n*-hexane for 15 min and the organic phases were merged. Subsequently, samples were centrifuged for 4 min (4300 rcf) and the organic phases were transferred to new 10 mL glass centrifuge vials. Samples were evaporated under a gentle nitrogen stream down to 50 μL , were transferred to measuring vials with 250 μL glass inserts, and stored at $-20\text{ }^{\circ}\text{C}$ until chemical analysis. All glassware was annealed before use ($7.1\text{ }^{\circ}\text{C/min}$ to $500\text{ }^{\circ}\text{C}$ (4.5 h)).

Chemical analysis using GC-APCI-MS/MS

Samples were measured on a GC-APCI-MS/MS instrument (Agilent gas chromatograph 7890B coupled to an Agilent triple quadrupole mass spectrometer 6495 using APCI). For chromatographic separation, a fused silica Rxi guard column (5 m, 0.25 mm i.d., Restek) and a fused silica Rtx-5MS column (30 m, 0.25 μm film thickness, 0.25 mm i.d., Restek) were used. The oven temperature gradient was as follows: $100\text{ }^{\circ}\text{C}$ (1 min), $25\text{ }^{\circ}\text{C/min}$ to $150\text{ }^{\circ}\text{C}$, $10\text{ }^{\circ}\text{C/min}$ to $300\text{ }^{\circ}\text{C}$ (3 min). Each run was performed in backflush mode which lowers the maintenance interval. Splitless injections of 5 μL using a deactivated liner (borosilicate glass, 4 mm i.d., Restek) were performed at $250\text{ }^{\circ}\text{C}$. Helium (99.999%, Carbagas, Switzerland) was used as carrier gas with a flow rate of 3 mL min^{-1} . The transfer capillary to the APCI interface was constantly maintained at $280\text{ }^{\circ}\text{C}$, and N_2 ($\sim 99\%$, N_2 generator) was used as source gas (11 L min^{-1}). The source gas temperature was set to $150\text{ }^{\circ}\text{C}$, the APCI corona discharge current to 1 μA , the capillary voltage to 1000 V, and the ion funnels to 100/40 V for high-pressure/low-pressure RF (radio frequency).

Ionization was performed in positive ionization mode using water as a modifier to promote the formation of protonated molecular ions $[\text{M} + \text{H}]^+$ (see Portoles et al. [23]). Therefore, nanopure water was pumped with a flow rate of $50\text{ } \mu\text{L min}^{-1}$ into a small uncapped vial placed in the ionization source. The mass spectrometer was operated in dynamic multiple reaction monitoring (MRM) mode with a cycle time of 250 ms and the mass resolution was set to 1.2 Da (quadrupole 1 and 3, wide isolation mode). N_2 (99.999%, Carbagas, Switzerland) was used as collision gas. For all analytes and ISTDs, at least two transitions were measured, from which the most sensitive

transition was used as quantifier and the remaining transition(s) as qualifier(s). Data acquisition was performed without time filtering (smoothing). The raw data are slightly smoothed afterwards in the Masshunter Qualitative or Quantitative Analysis software B.07.00, Agilent (Gaussian, function width = 15, Gaussian width = 2).

Quantification

To construct a calibration curve, 200 mL of tap water was transferred into 200 mL glass volumetric flasks and ISTD mix solution was added. Subsequently, analyte standard mix solution was added at different concentrations (1.25, 12.5, 25, 125, 250, 1250, 2500, 12,500, and 25,000 pg L^{-1}), and samples were extracted and analyzed as described in the “[Sample preparation of surface water by liquid–liquid extraction](#)” and “[Chemical analysis using GC-APCI-MS/MS](#)” sections.

Quantification was based on internal standard calibration (MassHunter Quantitative Analysis software B.07.00, Agilent). Peak area ratios were formed between the area of the analyte and the area of its corresponding ISTD and were compared to those in the respective calibration curve. When diastereomers were chromatographically separated for individual analytes, all peaks were integrated and summed. Matching ISTDs were available for bifenthrin (bifenthrin-d5), chlorpyrifos-methyl (chlorpyrifos-methyl-d6), α -cypermethrin (trans-cypermethrin-d6), deltamethrin (deltamethrin-d5), esfenvalerate (fenvalerate-d7), and etofenprox (etofenprox-d5). For the remaining analytes, relative recoveries (accuracies) were calculated based on all available ISTDs. The ISTD which resulted in the best relative recovery was selected for the final quantification (see [ESM.B](#)). Calibration curves were acquired at the beginning and at the end of each measuring sequence and were obtained using a linear least square regression with a weighting factor of $1/x$. Calibration curves were linear for all analytes in the used calibration range ($R^2 > 0.98$) except for allethrin, imiprothrin, and tetramethrin (see the “[Liquid–liquid extraction](#)” section).

Method validation

The optimized method was validated by determining relative recoveries (intra-day and inter-day accuracies), absolute recoveries of nearly the entire sample preparation covering LLE and phase separation (absolute recoveries_{LLE}), precisions (intra-day and inter-day), instrumental LOQs (ILOQs), and MLOQs. In order to determine inter-day relative recoveries and inter-day precisions, additional recovery experiments were carried out after several months.

Recoveries and precisions

To determine relative recoveries, 200 mL surface water was spiked with analyte standard mix solution to a concentration of 500 pg L⁻¹ and extracted. Additionally, 200 mL unspiked surface water was extracted to determine background contamination of the used surface water. For both sample types, ISTD mix solution was added before extraction and samples were analyzed and quantified according to the methods described in “[Chemical analysis using GC-APCI-MS/MS](#)” and “[Quantification](#)” sections. Absolute recoveries_{LLE} were determined to track analyte losses that might occur during LLE and phase separation. Therefore, 200 mL surface water was spiked with analyte mix solution to a concentration of 500 pg L⁻¹ (final extract concentration of 2 ng mL⁻¹) and ISTD mix solution was added after the extraction but before evaporation with nitrogen. Additionally, unspiked surface water was extracted and spiked with analyte mix solution (final extract concentration of 2 ng mL⁻¹) and ISTD mix solution after the extraction but before evaporation with nitrogen. Background contamination was determined by analyzing unspiked surface water and adding ISTD mix solution after the extraction but before evaporation with nitrogen. Samples were analyzed and the peak area ratios of each analyte in the surface water sample (analyte standard mix solution spiked before extraction, ISTD mix solution spiked after extraction) were compared to those, where analyte standard mix solution and ISTD mix solution were spiked after extraction taking background contamination into account. All samples were prepared in triplicate to determine method precision.

Method and instrumental limits of quantification

MLOQs were estimated from calibration standards that were prepared in tap water and went through the entire sample preparation (see the “[Sample preparation of surface water by liquid–liquid extraction](#)” section). MLOQs were defined by the concentration of the calibration standard that yielded analyte peaks with signal-to-noise (S/N) ratios of at least 10 and 3 for the quantifier and qualifier ion, respectively. ILOQs were determined by directly injecting analyte standards in pure solvent and the resulting peaks had to fulfill the same S/N criteria. Analyte Protection Mix (see [ESM.C](#)) composed of sugar and sugar derivatives was added to calibration standards prepared in pure solvent that were directly injected into the GC system without previous sample preparation. Calibration standards in pure solvent contained less matrix components compared to the extracts from surface water or tap water. Therefore, Analyte Protection Mix was used to block active sites in the GC system, thereby improving the chromatographic peak shape.

Average qualifier ion to quantifier ion ratios (qualifier ratios) based on peak areas were calculated for each

analyte using the qualifier ratios of all calibration standards. The qualifier ratio of each analyte in all environmental samples was compared to the average qualifier ratio and had to fall in a specified range according to the EC Directive 2002/657/EC [31].

Blank samples without ISTD (only tap water) and with ISTD (tap water and ISTD) were prepared simultaneously with environmental samples and went through the whole sample preparation. When low signals of analytes were found in blank samples, the highest peak area detected in the blank samples was doubled and compared to the analyte peak areas detected in the calibration standards. MLOQs for analytes present in the blank samples were then defined by the concentration of a calibration standard that exhibited the closest higher peak area compared to those in the blank samples.

Stability of pyrethroids and organophosphates during storage of surface water samples

To investigate the stability of pyrethroids in surface water samples during storage, six times 500 mL of surface water was spiked to a concentration of 500 pg L⁻¹ with analyte standard mix solution in 1 L glass vessels and samples were either stored at +4 °C or at –20 °C in the dark. After 1 week of storage, vessels were thoroughly shaken for 2 min, and 200 mL of water sample were transferred to 200 mL glass volumetric flasks and ISTD mix solution was added. Initial pyrethroid concentrations in the spiked surface water samples were determined and used for comparison. All samples were prepared in triplicate, and samples were extracted and analyzed as described in the “[Sample preparation of surface water by liquid–liquid extraction](#)” and “[Chemical analysis using GC-APCI-MS/MS](#)” sections.

Ecotoxicological risk assessment

To assess the acute risk to aquatic organisms, the measured environmental concentrations of each detected substance of the 3.5-day composite samples were compared to the corresponding acute quality criteria (MAC-EQS: maximum acceptable concentration environmental quality standard). To evaluate the risk to aquatic organisms towards long-term pollution, time-weighted average concentrations were calculated. Therefore, the concentrations of each detected substance of four subsequent 3.5-day composite samples were averaged resulting in 14-day composite samples and were compared to the corresponding chronic quality criteria (AA-EQS: annual average environmental quality standard). According to Spycher et al. [32], “moving averages” of four 3.5-day composite samples were formed by averaging the concentrations of equal time windows after the initial formed average of the first four samples. In this way, the effect of different 14-day time intervals was investigated.

Results and discussion

Liquid–liquid extraction

Based on existing solvent-based extraction methods of pyrethroids from water samples using a water-immiscible organic solvent (see reviews by Albaseer et al. [17] and Feo et al. [16]), LLE (enrichment factor of 4000 \times) with *n*-hexane was tested in two cycles. Absolute recoveries_{LLE} were between 67 and 114% (see Table 2) showing sufficient extraction efficiencies.

The more polar pyrethroids allethrin, imiprothrin, and tetramethrin could not be quantitatively determined because no linear calibration curves were obtained. These pyrethroids differ in their structures and have lower log K_{ow} values when compared to the remaining pyrethroids (see log K_{ow} values in Table 2). According to Hladik and Kuivila [33], allethrin and tetramethrin are mainly present in the dissolved fraction and do not sorb to sediments. However, the extraction efficiency of allethrin and tetramethrin from the water phase to the organic *n*-hexane phase does not seem to be hindered, since absolute recoveries_{LLE} of these compounds are $\sim 80\%$ (see Table 2). No absolute recoveries_{LLE} could be determined for imiprothrin because at a concentration of 500 pg L⁻¹, no chromatographic peaks were visible in the MRM chromatograms of the surface water samples. Reasons can be divers, such as an impaired extraction efficiency and/or suboptimal GC conditions. In general, especially for the more polar pyrethroids, the peak shapes clearly improved with increasing matrix load in the final extracts. Furthermore, no isotopically labeled ISTDs were available for the more polar pyrethroids, which would have added to the linearity of the calibration curves.

However, the focus of this method was on pyrethroids that are applied as plant protection products. Therefore, the substance spectrum of the developed method was regarded as satisfactory since in Switzerland, the more polar pyrethroids allethrin, tetramethrin, and imiprothrin are only in use as biocides (see Table 2).

Optimization of APCI-MS/MS parameters

The effects of different source parameters such as gas temperatures (80, 150, and 290 °C), APCI corona discharge currents (1 and 2 μ A), and capillary voltages (500, 1000, 2000, 3000, and 4000 V) were evaluated regarding peak intensities. The highest peak intensities were achieved using a source gas temperature of 150 °C, an APCI corona discharge current of 1 μ A and a capillary voltage of 1000 V. In addition, different parameters for the ion funnels (170/80, 150/60, and 100/40 V positive high pressure RF/positive low pressure RF) were tested but they did not significantly change the signal intensities; therefore, the final measurements were performed with 100/40 V (for further details on the different source parameters, refer to

ESM.D). Furthermore, the positioning of the ion transfer capillary with respect to the mass spectrometer inlet and the corona discharge needle is critical with regard to signal intensities and requires an accurate adjustment (see the picture in ESM.E).

In a further step, the influence of water on the formation of $[M + H]^+$ during ionization was tested. Therefore, according to Portoles et al. [23], water was placed as a modifier in the ionization source which clearly favored the formation of $[M + H]^+$ over M^+ during ionization (see ESM.F). Only for bifenthrin, etofenprox, and permethrin, fragment ions were used as precursor ions because of the lack of $[M + H]^+$, which has been previously observed by Portoles et al. [23] for bifenthrin and permethrin. Furthermore, analyte peak intensities of precursor ions were compared in the presence and absence of water. Peak intensities were higher using water as a modifier with the exceptions of bifenthrin, tefluthrin, and esfenvalerate (see ESM.F). Consequently, the use of water increased the sensitivity and the selectivity for most analytes by promoting the formation of $[M + H]^+$. Additionally, when using water as a modifier, ionization is independent of variations in humidity in the lab and remains constants during each instrument run.

Next, product ions and collision energies were optimized for all analytes and ISTDs in initial experiments by injecting individual analytes and ISTDs in *n*-hexane. Therefore, product ion scans of precursor ions were run with different collision energies (10, 20, 30, 40, and 50 eV) to select the most intense product ions depending on the applied collision energies. Each analyte and ISTD was injected individually and measured with the final MRM method. Subsequently, the MRM chromatograms of the individually injected analytes and ISTDs were evaluated with regard to signals occurring on the MRM chromatograms of the other analytes and ISTDs included in the method. In this way, it was ensured that no interfering signals from non-target substances appeared in the MRM chromatograms of each analyte and ISTD. Finally, at least two transitions were measured for each analyte and ISTD, from which the most sensitive transition was selected as quantifier and the remaining transition(s) as qualifier(s) and the qualifier ratio had to fall in the specified range. Table 1 summarizes the optimized conditions used for GC-APCI-MS/MS analysis.

Stereoisomers of pyrethroids—consequences for identification and quantification

In total, 12 relevant and frequently used apolar pyrethroids were analyzed. Some of the deployed pyrethroid reference standards (λ -cyhalothrin, esfenvalerate, α -cypermethrin, τ -fluvalinate, and deltamethrin) only included a reduced number of possible stereoisomers because of commercial availability or because only selected stereoisomers serve as active ingredients in plant protection products or biocides that are approved in Switzerland. This use of isomer-enriched reference standards can lead to different challenges:

Table 1 Optimized GC-APCI-MS/MS conditions for all target analytes and ISTDs

Compound	Retention time [min] ^a	Precursor ion [M + H] ⁺ [m/z] ^b	Product ions [m/z] ^c (collision energy [eV])	Dwell time [ms]
Analytes				
Empenthrin	6.6 / 6.7	275.2	173.0 (20) , 229.0 (10)	120
Tefluthrin	7.6	419.1	177.0 (40) , 325.0 (10)	79
Chlorpyrifos-methyl	8.3	321.9	125.0 (30) , 289.9 (20)	42
Chlorpyrifos	9.2	349.9	198.0 (20) , 97.0 (40), 294.0 (10)	31
Bifenthrin	13.2	<i>181.0[#]</i>	165.0 (30) , 115.0 (40)	26
Phenothrin	13.5 / 13.6	351.2	183.0 (20) , 129.0 (50)	29
λ-Cyhalothrin	14.0 / 14.2	450.1	225.0 (10) , 141.0 (40)	37
Acrinathrin	14.1 / 14.3	542.1	181.0 (40) , 289.0 (20), 93.0 (50)	38
Permethrin	14.9 / 15.0	<i>355.0[#]</i>	261.0 (20) , 319.0 (10), 355.0 (10)	42
α-Cypermethrin	15.7 / 15.9	416.1	191.0 (10) , 127.0 (20)	25
Etofenprox	16.0	<i>359.2[#]</i>	183.0 (20) , 189.0 (20)	32
Esfenvalerate	16.6 / 16.8	420.1	167.0 (10) , 125.0 (50)	28
τ-Fluvalinate	16.8 / 16.9	503.1	208.0 (10) , 181.0 (20)	28
Deltamethrin	17.1 / 17.3	504.0	278.9 (10) , 172.0 (30)	34
Internal standards (ISTDs)				
Chlorpyrifos-methyl-d6	8.3	327.9	131.0 (30) , 293.0 (20)	42
Bifenthrin-d5	13.3	<i>186.0[#]</i>	170.0 (40) , 118.0 (50)	26
trans-Cypermethrin-d6	15.8 / 16.0	422.1	96.0 (40) , 133.0 (40)	25
Etofenprox-d5	16.1	<i>364.0[#]</i>	183.0 (30) , 194.0 (10)	32
Fenvalerate-d7	16.7 / 16.9	427.2	174.0 (20) , 127.0 (50)	28
Deltamethrin-d5	17.2 / 17.4	509.0	279.0 (30) , 172.0 (10)	34

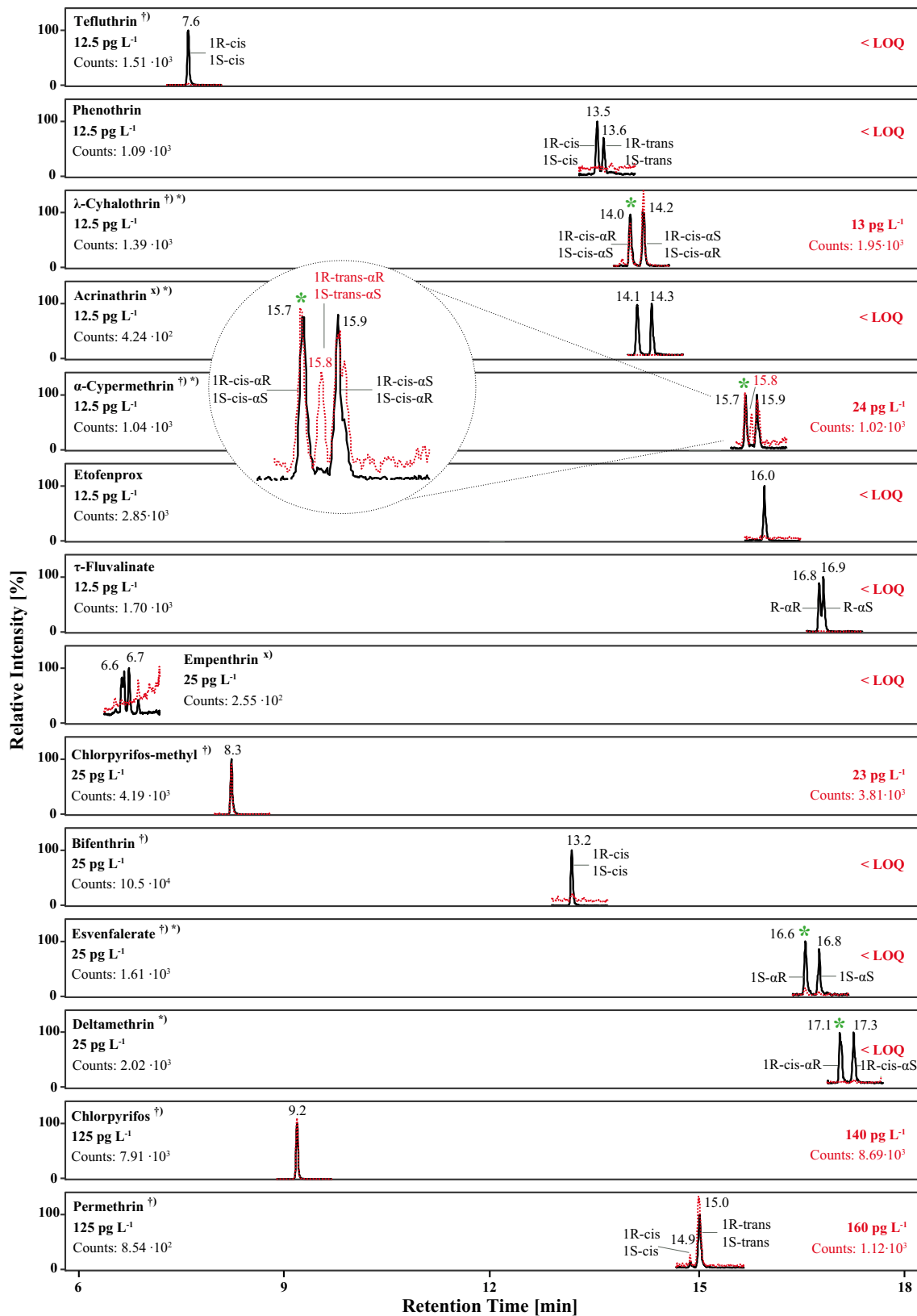
^a When several retention times are listed, they refer to the separation of stereoisomers^b Fragment precursor ions are italicized and are marked with a #^c Product ions that are part of the quantifier transition are marked in bold

- (I). A unique peak assignment has to be ensured even when the following occurs:
- Different isomerization processes occur in the environment compared to the lab
 - Interfering peaks occur in environmental samples
 - Additional stereoisomers are approved as active ingredients in plant protection products and/or biocides leading to additional stereoisomer peaks in the MRM chromatograms of environmental samples
- (II). The instrumental response needs to be similar for all stereoisomers of one pyrethroid. Thus, a reliable quantification can be ensured in case additional stereoisomer peaks occur in the MRM chromatograms of environmental samples.

Therefore, it was first tested if all stereoisomers of one pyrethroid exhibit a similar instrumental response. This has been confirmed by quantifying cypermethrin (all stereoisomers) in a certified reference standard using α-cypermethrin,

which resulted in a recovery of 110%. Additionally, peak areas of similar concentrations of esfenvalerate (1 stereoisomer) and fenvalerate (4 stereoisomers) as well as λ-cyhalothrin (2 stereoisomers) and cyhalothrin (8 stereoisomers) were compared and peak areas varied by less than 20%. Thus, no issues occurred in quantification even when the reference standards were composed of a reduced number of stereoisomers.

Second, all separated stereoisomer peaks of one pyrethroid had to be unequivocally assigned to each pyrethroid, which is necessary for a reliable peak integration and consequently for a proper quantification. For example, cypermethrin displayed an additional peak in the MRM chromatogram of the surface water samples when compared to the α-cypermethrin reference standard (see Fig. 2). To assign all cypermethrin peaks clearly, different isomer-selective cypermethrin standards and a reference standard of cypermethrin containing all stereoisomers were measured. Figure 1b–g shows the MRM chromatograms of α-, β-, θ-, and ζ-cypermethrin and of cypermethrin



◀ **Fig. 2** MRM chromatograms showing the quantifier transitions of all analytes in calibration standards at the MLOQ (black, continuous) and in surface water samples (red, dashed). Calibration standards and surface water samples went through the entire sample preparation method. For analytes that were detected in the surface water samples, a surface water sample was selected that exhibited analyte concentrations around the corresponding MLOQ; otherwise, a surface water sample without positive detection is shown. Analytes are sorted with increasing MLOQ and within one concentration by increasing retention time. Note that for analytes marked with a dagger sign, the MLOQ is a result of low-level blank contamination and the actual instrumental sensitivity can be much lower (see ILOQ in Table 2). Signals in the calibration standards are displayed as relative intensities (normalized to 100%), and signals in the surface water samples are displayed relative to the highest intensity in the corresponding calibration standard. Where possible, all separated diastereomers are labeled per pyrethroid based on own or literature data [8, 20, 34, 35]. For acrinathrin and empenethrin that are marked with letter x, the single isomer peaks could not be assigned because of the high number of eight stereoisomers per substance. Pyrethroid peaks that are a result of isomerization in ethanol at the α -carbon position are marked with a green asterisk, and all pyrethroids carrying the α -carbon are labeled with a black asterisk. Note that a different GC column of the same Rtx-5MS type was used for the measurement of the isomer-selective cypermethrin reference standards displayed in Fig. 1, explaining why the retention times of the cypermethrin peaks are slightly shifted

that contains all stereoisomers. Cypermethrin has three chiral centers and thus comprises eight stereoisomers (see Fig. 1a). The different cypermethrin stereoisomers are classified into different subgroups, i.e., α -cypermethrin (2 cis isomers: 1R-cis- α S, 1S-cis- α R), β -cypermethrin (2 cis isomers: 1R-cis- α S, 1S-cis- α R and 2 trans isomers: 1R-trans- α R, 1R-trans- α S), θ -cypermethrin (2 trans isomers: 1R-trans- α S, 1S-trans- α R), and ζ -cypermethrin (4 α S isomers: 1R-cis- α S, 1R-trans- α S, 1S-cis- α S, 1S-trans- α S). The separation of the four diastereomers (enantiomeric pairs) of cypermethrin is displayed in Fig. 1g with the elution order cis, trans, cis, trans, which has been previously observed in other studies using similar apolar GC columns [20, 34]. By separately injecting the isomer-selective reference standards of α -, β -, θ -, and ζ -cypermethrin (see Fig. 1b–f), all peaks in the MRM chromatogram of cypermethrin that comprises all stereoisomers could be assigned to individual stereoisomers (Fig. 1g). No baseline separation was achieved for the cis and trans diastereomers eluting after 16.45 and 16.49 min. Consequently, the additional cypermethrin peak observed in the MRM chromatogram of the surface water samples with a retention time of 15.8 min (see Fig. 2) belongs to cypermethrin and is composed of the stereoisomers 1R-trans- α R and 1S-trans- α S. For final identity confirmation, the qualifier ratio of each baseline separated peak of cypermethrin in the surface water samples was compared separately to the corresponding peak in the MRM chromatogram of a reference standard of cypermethrin containing all stereoisomers. The qualifier ratio of each cypermethrin peak in the surface water samples stayed within the allowed range ($\pm 30\%$ for analytes, i.e., cypermethrin with qualifier ratios > 10

to 20%) according to the EC Directive 2002/657/EC [31], thereby confirming that each cypermethrin peak in the MRM chromatogram of a surface water sample actually belongs to cypermethrin.

Figure 1b illustrates the isomerization of α -cypermethrin when stored in ethanol. An additional peak occurred at a retention time of 16.28 min when compared to α -cypermethrin prepared in *n*-hexane (see Fig. 1c). The comparison of the retention time of the additionally observed peak of α -cypermethrin in ethanol with the elution pattern of the peaks present for cypermethrin (all stereoisomers) showed that the isomerization of α -cypermethrin took place at the α -carbon position containing the cyano group and that the chiral centers at the cyclopropyl ring were unaffected (see Fig. 1a, b, g). Thus, in ethanol, the two stereoisomers 1R-cis- α S and 1S-cis- α R of α -cypermethrin were converted to 1R-cis- α R and 1S-cis- α S, resulting in four instead of the initial two stereoisomers. Isomerization in ethanol at the α -carbon position was also observed for deltamethrin, λ -cyhalothrin, and esfenvalerate (see Fig. 2). Therefore, especially for pyrethroids carrying the α -carbon, isomerization is critical and polar protic solvents such as water and alcohols should be avoided, whereas stereoisomers remained stable in aprotic solvents, such as in *n*-hexane or ethyl acetate [35–40].

Overall, the use of pyrethroid reference standards composed of all stereoisomers is recommended as a calibration method. Thereby, a unique peak assignment in environmental samples can be guaranteed and, with the prerequisite of comparable instrumental responses of all stereoisomers of one pyrethroid, a reliable quantification can be performed. When special interest is given to the identification and quantification of single stereoisomers in environmental samples, care should be taken not to use protic solvents for the preparation of reference standards. However, assessing the initial isomer composition in environmental samples is in any case difficult because of naturally occurring isomerization processes after entry into the environment.

Method validation

Quality control parameters (MLOQs, ILOQs, precisions (intra-day and inter-day), absolute recoveries_{LLE}, and relative recoveries (intra-day and inter-day)) were determined for the optimized method.

In general, MLOQs were very low and varied between 12.5 and 25 pg L⁻¹ for most analytes which is a prerequisite for the assessment of ecotoxicological relevant concentrations (see MLOQs and EQSs in Table 2). Chlorpyrifos and permethrin exhibited MLOQs of 125 pg L⁻¹ resulting from low-level contamination in the blank samples (see calculation of MLOQs in the “Method validation” section for the case of low signals in blank samples). Especially MLOQs resulting from small blank contaminations can vary for each day of

Table 2 Physico-chemical properties, quality control parameters, limits of quantification in surface water (MLOQ), environmental quality standards (EQSs), and environmental concentrations measured in Swiss surface waters

Compound ^a	Number of stereoisomers ^b	Log K_{ow} ^c	Intra-day relative recovery ^d [%]	Absolute recovery _{LLE} ^d [%]	ILOQ [pg L ⁻¹]	MLOQ ^e [pg L ⁻¹]	AA-EQS ^f [pg L ⁻¹]	MAC-EQS ^f [pg L ⁻¹]	Surface water concentration [pg L ⁻¹]	Approval in Switzerland ^g
λ -Cyhalothrin ^h	2 2 4	6.8	91 ± 5	94 ± 12	0.025	12.5 ⁱ	22 [12]	190 [12]	< LOQ–31,000	B, P
α -Cypermethrin ^h	2 2 4	6.6	93 ± 13	89 ± 1	0.25	12.5 ⁱ	30 [12] / 80 [11]	440 [12] / 600 [11]	< LOQ–980	B, P
Tefluthrin	4 2	6.5	99 ± 2	96 ± 9	0.25	12.5 ⁱ	16 [41] ^j	–	< LOQ	P
Phenothrin	4 4	6.0	107 ± 11	84 ± 9	0.25	12.5	1 [41] ^j	–	< LOQ	B
Acinathrin ^h	8 8	6.5	84 ± 15	89 ± 7	0.25	12.5	–	–	< LOQ	–
Etofenprox	1 1	7.1	110 ± 8	96 ± 12	0.025	12.5	5400 [42]	–	< LOQ	B, P
τ -Fluvalinate	2 2	6.9	93 ± 8	86 ± 8	0.025	12.5	–	–	< LOQ	–
Empenthrin	8 8	5.6	109 ± 5	112 ± 11	0.25	25	–	–	< LOQ	B
Bifenthrin	4 2	6.6	133 ± 3	67 ± 5	0.25	25 ⁱ	20 [43] ^j	10,000 [43]	< LOQ	B, P
Chlorpyrifos-methyl	–	4.3	100 ± 5	103 ± 3	0.025	25 ⁱ	1000 [12]	7300 [12]	< LOQ–140	P
Esfenvalerate ^h	1 1 2	6.2	100 ± 2	90 ± 7	0.025	25 ⁱ	100 [44]	850 [44]	< LOQ	B
Deltamethrin ^h	8 1 2	6.2	100 ± 2	104 ± 3	0.25	25	3 [45] ^j	310 [45]	< LOQ	B, P, VD
Chlorpyrifos	–	5.0	90 ± 22	114 ± 14	0.025	125 ⁱ	460 [12] / 30,000 [11]	4400 [12] / 100,000 [11] ^k	< LOQ–10,000	P
Permethrin	4 4	6.5	100 ± 11	80 ± 7	0.25	125 ⁱ	470 [46]	10,000 [46] ^k	< LOQ–650	B, VD
Allethrin	8 8	4.8	–	84 ± 11	0.25	–	–	–	–	B
Tetramethrin	4 4	4.7	–	77 ± 15	0.25	–	290 [41]	–	–	B
Imiprothrin	4 4	2.9	–	–	2.5	–	–	–	–	B

^a Analytes with corresponding ISTD are italicized^b Theoretical number of stereoisomers | number of stereoisomers in the technical product | number of stereoisomers due to isomerization in ethanol at α -carbon position containing the cyano group^c Experimental log K_{ow} values were taken from the Chemistry Dashboard, US Environmental Protection Agency (<https://comptox.epa.gov/dashboard> [47]). Only for acinathrin, τ -fluvalinate, and bifenthrin log K_{ow} values were predicted with MarvinSketch version 14.10.20.0 (<http://www.chemaxon.com/> [48])^d Spike level, 500 pg L⁻¹; number of replicates $n = 3$ ^e For the determination of MLOQ refer to the “[Method validation](#)” section^f For cypermethrin (mixture of all stereoisomers) and chlorpyrifos, additional EQSs (marked in *italic*) are listed that are included in the WFD^g Status in July 2017: P, plant protection product; B, biocide; VD, veterinary drug^h Pyrethroids with a chiral center at the α -carbon position containing the cyano group. Isomerization in ethanol at the α -carbon position was observed for λ -cyhalothrin, α -cypermethrin, esfenvalerate, and deltamethrin. For acinathrin, isomerization at the α -carbon position most likely also occurred but it could not be chromatographically shown since acinathrin already contained the mixture of all possible stereoisomersⁱ MLOQ was determined via blank samples because of low-level blank contaminations^j AA-EQS is below MLOQ^k Ad hoc MAC-EQS based on acute data compiled in Loos et al. [46]

sample preparation and the actual instrumental sensitivity can be much lower. For 8 out of 14 analytes, low signals were found in the blank samples (see Table 2 and [ESM.G](#)). During later sample preparations, signals in blank samples were strongly decreased (MLOQ for all substances between 12.5 and 25 pg L⁻¹) and were only present for 5 out of the 14 analytes. To test for possible sources of contamination, annealed glass vessels were extracted with *n*-hexane, and the extracts were analyzed. Chlorpyrifos, chlorpyrifos-methyl, and permethrin were detected with concentrations of approximately 10 pg L⁻¹, although annealing should lead to residue-free glass vessels. In addition, blank samples composed of nanopure water were prepared to rule out an initial contamination of the used tap water. Similar blank contaminations were observed compared to those in blank samples prepared in tap water. Blank samples with ISTD were compared with those without ISTD. Bifenthrin and esfenvalerate were only detected in blank samples with ISTD, indicating that the low-level blank contamination is a result of slightly contaminated matching ISTDs with the respective analyte. Based on the excellent instrumental sensitivity (see ILOQ in Table 2) of the analyzed substances, achieving completely clean blank samples remains challenging. Therefore, when dealing with ultra-sensitive methods such as GC-APCI-MS/MS, a thorough cleaning of lab equipment and instrumentation as well as the use of highly pure solvents and standards are required for achieving optimal MLOQs.

Differing environmental matrices between calibration standards (tap water) and environmental samples (surface water) potentially influenced the measurements. This possible bias was checked by comparing the S/N ratio and the peak areas in spiked surface water samples (spike level, 500 pg L⁻¹) with those in similar concentrated calibration standards prepared in tap water. Both sample types (tap water and surface water) were processed using the entire sample preparation method. Peak areas varied on average by ~40% between calibration standards and spiked surface water samples. This variation includes the volumetric uncertainty of the final sample volume of 50 µL. Furthermore, the possible matrix effects were evaluated by checking surface water samples in which analytes were detected at concentrations around their corresponding MLOQ. No signal suppression was observed in surface water samples for the positively detected pyrethroids and organophosphates (see Fig. 2). This result indicates that the ionization of analytes in the GC-APCI interface is not significantly influenced by matrix constituents under the selected conditions.

Intra-day precision was on average 8%, intra-day relative recoveries were between 84 and 133%, and analyte losses during the entire sample preparation method (absolute recoveries_{LLE}) were minimal resulting in absolute recoveries_{LLE} between 67 and 114% (see Table 2). Inter-day

precision was on average 12% and inter-day relative recoveries varied between 85 and 108% (for details refer to [ESM.HS1](#)). Additionally, surface water samples were spiked in duplicate to a concentration of 25 pg L⁻¹ to determine relative recoveries and precisions in the range of the MLOQ. Resulting relative recoveries were between 85 and 118% and precision was on average 10% (for details refer to [ESM.I](#)). However, for chlorpyrifos and permethrin, no recoveries at the MLOQ could be determined as chlorpyrifos was already present in the surface water samples with a concentration of 420 pg L⁻¹ (the spike level was too low) and for permethrin the spike level was < MLOQ (see Table 2).

These quality control parameters of the optimized method using LLE with a large enrichment factor of 4000× in combination with GC-APCI-MS/MS show that the method is ultra-sensitive and robust and is therefore suitable for the analysis of pyrethroids and organophosphates at ecotoxicological relevant concentrations.

Storage stability

Storage stability experiments showed that the storage of spiked surface water at +4 °C over a period of 1 week in the dark (as it was the case during sampling in the on-site cooled sampling device and during cooled shipping to the laboratory) can lead to analyte losses of on average 40%. Nearly no decrease in concentration was observed for the organophosphates, whereas empenhrin concentrations decreased up to 70%. Moreover, storage stability at -20 °C (storage temperature of surface water samples in the laboratory before analysis) was tested by freezing spiked surface water samples for 1 week in the dark. Storage at -20 °C showed analyte losses of on average 20%. Analyte concentrations remained constant for the organophosphates and tefluthrin, and the strongest decrease was observed for bifenthrin (~45%). Consequently, freezing seems to be the preferred method for almost all analytes during storage of aqueous samples. For further details on the storage stability of the single analytes, refer to [ESM.J](#). However, because of the observed analyte losses during storage at +4 °C and -20 °C, the ecotoxicological risk in the investigated surface water might be even higher.

The reasons for these concentration decreases during storage at +4/-20 °C in the surface water samples can be diverse, such as sorption to sample vessels. However, sorption has been shown to be a reversible process [33, 49] and in this study, all glass vessels containing water samples were shaken thoroughly before extraction. Additionally, sorption to glass vessels increases with decreasing dissolved organic carbon (DOC) and is therefore dependent on the DOC content in the water sample [33, 49]. The DOC content in the surface water samples used for the stability experiment in this study was ~2.2 mg L⁻¹ and Hladik and Kuivila [33] have observed that ~16–27% of selected pyrethroids were associated with

the glass bottle walls in waters containing 1 mg L^{-1} DOC. Moreover, agitation of water samples before extraction leads to a resuspension of particles. Without resuspension of analytes that are associated to particles, recoveries have been shown to decrease strongly since particle bound analytes are excluded from the aqueous phase [49]. Microbial degradation of analytes seems to be only important when stored at 4°C , since at -20°C microbial activity is limited and for some analytes the observed losses at -20°C are only slightly higher than the method precision (see ESM.J). Further studies are needed to distinguish between possible processes responsible for the observed analyte losses, especially at 4°C .

Application to environmental surface water samples

Out of the 14 analyzed substances, three pyrethroids (cypermethrin, λ -cyhalothrin, and permethrin) and both organophosphates (chlorpyrifos and chlorpyrifos-methyl) were detected in the investigated Swiss surface water samples taken between March and October 2017 (see Table 2 and Fig. 3). Chlorpyrifos was detected in nearly all samples (57/58), followed by λ -cyhalothrin (43/58), cypermethrin (19/58), chlorpyrifos-methyl (10/58), and permethrin (5/58). Peak concentrations were found between April and June for λ -cyhalothrin ($31,000 \text{ pg L}^{-1}$), chlorpyrifos ($10,000 \text{ pg L}^{-1}$), and cypermethrin (980 pg L^{-1}) which is in line with the main period of plant protection product application. By contrast, chlorpyrifos-methyl showed a different concentration pattern

over time. The highest concentrations were reached in the middle of March (140 pg L^{-1}), decreased until the beginning of May and disappeared afterwards, likely following the differing application times of this plant protection product in the catchment. Permethrin showed single detects between March and June. Besides permethrin, which is approved as a biocide and veterinary drug, all detected insecticides are permitted as plant protection products in Switzerland (see Table 2) and are registered for the application on vegetables (chlorpyrifos, cypermethrin, and λ -cyhalothrin), cereals (chlorpyrifos), orchards (chlorpyrifos, chlorpyrifos-methyl, and cypermethrin), and rapeseed (chlorpyrifos, chlorpyrifos-methyl, cypermethrin, and λ -cyhalothrin).

The measured concentrations in the original samples (3.5-day composite samples) were compared to the acute quality criteria. MAC-EQS exceedances were present for λ -cyhalothrin, chlorpyrifos, and cypermethrin in 10/58, 6/58, and 2/58 samples, respectively. Although the time of exceedance was rather short, MAC-EQSs were highly exceeded (see Fig. 3) indicating an acute risk for aquatic organisms during this time. To assess the risk of aquatic organisms towards long-term pollution, the averaged 14-day composite samples were compared to the chronic quality criteria. Since the averaged 14-day composite samples refer to calculated concentrations, they can exhibit concentrations below the corresponding MLOQ as observed for permethrin and chlorpyrifos-methyl (see Fig. 3). AA-EQSs were exceeded for all detected substances except for chlorpyrifos-methyl and permethrin (see

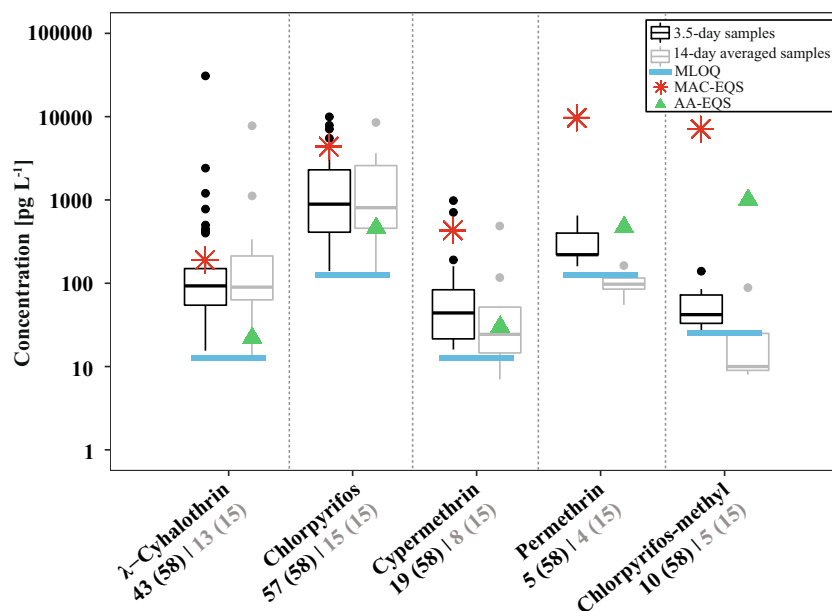


Fig. 3 Boxplots of the concentrations of the positively detected pyrethroids and organophosphates in the original 3.5-day composite samples (black) and in the averaged 14-day composite samples (gray) in comparison with their method limit of quantification (MLOQ) as well as acute (MAC-EQS) and chronic (AA-EQS) quality criteria. The

meaning of the numbers below the analyte names: number of detections in the 3.5-day composite samples (total number of analyzed 3.5-day composite samples) | number of detections in the 14-day averaged samples (total number of calculated 14-day averaged samples)

Fig. 3). Notably for chlorpyrifos and λ -cyhalothrin, AA-EQS exceedances were present for 11/15 and 12/15 averaged 14-day composite samples, respectively, thereby exceeding chronic quality criteria during nearly the entire sampling period. AA-EQS exceedances were not significantly influenced by the differently composed 14-day time intervals (“moving averages”) used to calculate averaged 14-day composite samples (overall percentage of AA-EQS exceedance with fixed starting value of 33% and of 35% with moving time intervals).

Our study shows the significance of utilizing ultra-sensitive analytical methods to detect pyrethroids and organophosphates at ecotoxicological relevant concentrations. Comprehensive pesticide screenings in surface waters and sediments have shown that pyrethroids and organophosphates are often the main drivers of the overall risk, thereby highlighting the importance including them into ecotoxicological risk assessment [50, 51].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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